Effect of Garlic, Stinky Bean, Dogfruit, Tomato Extracts, and N-acetylcysteine on Rats after 5 Gy Irradiation

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ABSTRACT

The formation of reactive oxygen species (ROS) and free radicals is the most important effect of radiation exposure on biological systems. Several studies have shown that several vegetables are proven to have beneficial effects to protect the body from free radical attacks. This current study was focused on exploring the capability of extracts of garlic, stinky bean, dog fruit, and tomato, as well as N-acetylcystein (NAC), in counteracting free radicals induced by gamma irradiation with a dose of 5 Gy. Seven treatments on male rat were as follow: A (control), B (5 Gy), C (garlic + 5 Gy), D (stinky bean + 5 Gy), E (dog fruit + 5 Gy), F (tomato + 5 Gy) and G (NAC + 5 Gy). The rats were irradiated 8 days after the supplement had been given. Detection of malondialdehyde (MDA), glutathione (GSH), glutathione peroxidase (GPx), catalase (CAT), and 8-hydroxy-2-deoxyguanosine (8-OHdG) was done by biochemical assays, and γ-H2AX foci were counted by immunofluorescence assay to the lymphocytes and plasma samples. The results showed that gamma irradiation with a dose of 5 Gy caused increases in the level of MDA, 8-OHdG, and γ-H2AX foci while decreases were recorded in the level of GSH, GPx, and CAT ($p < 0.05$). The treatment of garlic, dog fruit, and tomato extracts and NAC reduced free radicals significantly. In conclusion, the tomato has the best ability to overcome free radicals due to gamma irradiation among the treatments in the experiment.

INTRODUCTION

Ionizing radiation has become an important part of medicine. It is used for both diagnostic and therapeutic purposes. Operators in radiotherapy are radiation workers at risk of radiation exposure [1]. Biological damage to normal cells is the main occupational health problem for radiation workers [2].

Gamma irradiation removes electron randomly and leads to the formation of free radicals [3]. It will remove the electron from surrounding molecules, especially protein, DNA, and lipid molecules, and create a group of reactive oxygen species (ROS). On the lipids, the irradiation attacks carbon double bond(s), especially in polyunsaturated fatty acids (PUFAs). The final products are lipid peroxides which can be detected as malondialdehyde (MDA). The body has a natural defense system mechanism in the form of endogenous antioxidants, namely superoxide dismutase (SOD), glutathione (GSH), glutathione peroxidase (GPx), and catalase (CAT), that neutralize free radicals and accelerate their degradation to prevent macromolecule damage. However, if the endogenous antioxidant

is insufficient, the body needs exogenous antioxidants [4].

On the DNA, radiation exposure produces DNA adduct in the form 8-hydroxy-2'-deoxyguanosine (8-OHdG), which is the main form of oxidative DNA damage biomarker [5]. The other important effect of radiation exposure was initiation of DNA double-strand breaks (DSBs) which are able to induce mutations, thus disrupting genomic integrity. The damaged histone is called γ-H2AX focus and is known to represent DNA DSBs as a biomarker for radiation exposure [6].

Several studies have shown that several vegetables protect the body from free radical. Garlic (*Allium sativum* L.) extract has strong antioxidant activity by organosulfur compounds which are soluble in water, such as S-allylcysteine and S-allylmercaptocysteine [7]. Stinky bean (*Parkia speciosa* Hassk.) contains phenolic compounds; it also contains cyclic polysulfides, namely, hexathionine, tetrathiane, trithiolane, pentathiopane, pentathiocane, and tetrathiepane, that exhibit antioxidant activity [8]. Meanwhile, the dogfruit or djenkol *(Archidendron jiringa)* contains djenkolic acid which has a similar structure to the cystine amino acid and hence has an ability to prevent lipid peroxidation [9]. Tomato (*Solanum lycopersicum* L.) contains carotenoid compound lycopene that is a powerful antioxidant and can protect the body against cancer of the prostate gland [10]. Besides the active compound, the vegetable also contains vitamin C and various phenolic compounds. N-acetylcysteine (NAC) is a derivative of cysteine which has antioxidant activity and is often used as a standard antioxidant [11].

The purpose of this study was to explore the capability of garlic, stinky bean, dog fruit, and tomatoes in protecting against gamma radiation, using NAC as reference.

EXPERIMENTAL METHODS

Materials

Garlic, stinky beans, dog fruits, and tomatoes as natural ingredients were obtained from Indonesian Farmers Stores (Fig. 1), while N-acetylcysteine was purchased from Sigma-Aldrich (product number A7250).

Male Wistar rats (*Rattus norvegicus*) 10-12 weeks old of age (170-250 grams) from an inbred colony (obtained from the Animals Laboratory, Ministry of Health, Indonesia) were used for our study. Animals were maintained under controlled conditions (temperature 21-24 °C) and light (12 h dark, 12 h bright) in the Integrated Animals

Laboratory, National Nuclear Energy Agency of Indonesia (BATAN). The rats were fed with a commercial balanced diet *ad libitum* and had a free access to tap water. All the animals were weighed regularly once a week.

(a) (b) (c) (d)
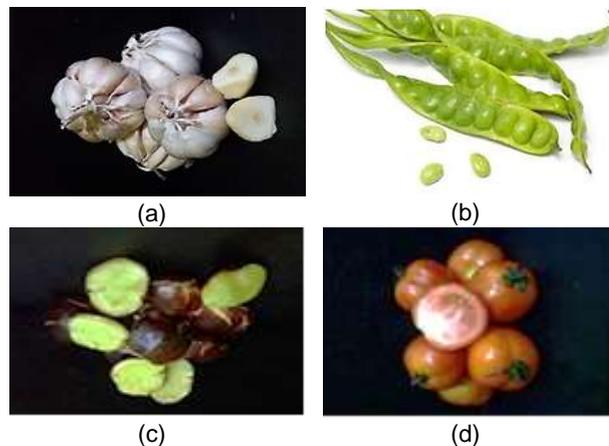
Fig. 1. Natural ingredients as antioxidant materials. (a) Garlic (*Allium sativum* L.), (b) Stinky bean (*Parkia speciosa* Hassk.), (c) Dogfruit (*Archidendron jiringa* (Jack) I.C. Nielsen.), (d) Tomato (*Solanum lycopersicum* L.)

Experimental design and irradiation

This study had obtained ethical approval from the Ethics Committee of the Faculty of Medicine, University of Indonesia, No. 0021/UN2.F1/ETIK/2019 on January 7, 2019. Twenty-eight male Wistar rats were divided into seven groups, each consisting of four rats. The groups were denoted as group A through G. Those groups, and their associated treatments, are as follows: A (control, without any treatment), B (5 Gy irradiation), C (garlic extract + 5 Gy), D (stinky bean extract + 5 Gy), E (dog fruits extract + 5 Gy), F (tomato extract + 5 Gy), and G (NAC + 5 Gy). All of the groups C to G were treated with their respective extracts or NAC for eight consecutive days. For all groups, the 5-Gy total body irradiation was given on the ninth day, by using the ^{60}Co IRPASENA device at the Center for Application of Isotope and Radiation (PAIR-BATAN) with a single dose of 5 Gy and a dose rate of 1 Gy/min. The ^{60}Co source distance to the sample was 108 cm.

Each rat was kept in a perforated acrylic container. The irradiated rats were placed in a rotating platform to ensure an equal dose to all tissues.

Preparation of materials

Garlic, stinky beans, and dogfruits were weighed to 100 grams each and homogenized by

using a blender. Each of the homogenites was placed in white gauze and squeezed to obtain the extract. Tomatoes were boiled until soft, then grated to obtain a liquid extract. Then, every extract was aliquoted to 15 ml in glass bottles and store at -20 °C. The doses of garlic, stinky bean, dogfruit, and tomato extracts were 125 milligram per kilogram body weight (mg/kgBW) [12], 400 mg/kgBW [13], 10 mg/kgBW [9], and 2.5 mg/kgBW [14], respectively, while NAC was given at 150 mg/kgBW [15]. To adjust the maximum capacity of the rat stomach (5 ml), the extract was then dissolved in aquadest to a volume of 2 ml. The extracts were administered to rats by oral gavage.

Isolation of lymphocytes and plasma

Prior to blood collection, the animals were anesthesized using 75-100 mg/kg of ketamine-xylazine injected intraperitoneally. Then, the thoracic cage was opened and the blood was removed directly using 10 mL heparin tube. The blood was placed into the heparin tube for separation of the cell and the plasma. The isolation procedure followed was, with some modifications, as previously described by Kurnia *et al*., Chua *et al*., and Mir *et al*. [16-18]. The heparinized blood samples were centrifuged at 3000 rpm for 15 minutes. Then, 200 µL of plasma for MDA and GSH assays were separated and stored at 4 °C for the subsequent steps of the experiment. The supernatant plasma was removed and the precipitated cells were placed in a tube containing 3 mL phosphate-buffered saline (PBS). The cell suspensions were layered carefully onto 3 mL volume of lymphocytes-separating medium (Histopaque 1077) in a centrifuge tube, centrifuged for 30 minutes at 1500 rpm. The lymphocytes appear with a whitish color, between the plasma and histopaque layers. The lymphocyte cells are transferred into centrifuge tubes containing 5 ml PBS, then centrifuged for 15 minutes at 1500 rpm. The supernatant was removed, and the sediments (lymphocytes) were resuspended in 3 mL RPMI medium, followed by another centrifugation at 1000 rpm for 15 minutes. Afterward, the supernatant was removed, and cryoprotectants and phenylmethylsulphonyl-fluoride (PMSF) in a ratio of 1:1 were added to the lymphocytes. Each measurement of MDA, GSH, CAT, GPx, 8-OHdG, and H2AX foci 500 ml of lymphocytes suspension are added respectively and stored at -80 °C for use in the subsequent experiments.

Measurement of lymphocytes and plasma protein concentration

Lymphocytes were frozen and thawed at -20 °C and 100 °C three times. The absorbance of the lymphocyte extract and, separately, plasma protein were measured at 280 nm. The concentrations of lymphocytes and plasma protein were calculated using the formula obtained from the bovine serum albumin (BSA) standard curve.

MDA assay

The MDA concentration was measured according to the method of Wills [19]. The MDA concentration was expressed in total protein concentration.

GSH assay

GSH was assayed according to the method of Schmitt *et al*. [20]. The GSH concentration was expressed in total protein concentration.

CAT specific activity

A total of 50 µL of lymphocyte lysate was added into a spectrophotometric cuvette. Then, 950 µL of H_2O_2 was added. The mixture was shaken manually and the absorbance was read at 210 nm. The specific activity of CAT was calculated by dividing CAT activity with lymphocyte lysate protein concentration [21].

GPx specific activity

The assay was performed according to the GPx Kit (Randox, RS505). The method is based on a technique described by Vega *et al*. [22]. Briefly, GPx catalyzes the oxidation of reduced glutathione by cumene hidroperoxide. In the presence of glutathione reductase and the reduced nicotionamide adenine dinucleotide phosphate (NADPH). The oxidized glutathione, or glutathione disulfide (GSSG), is immediately recycled to the reduced form with a concomitant oxidation of NADPH to NADP+. The decrease of NADPH absorbance was rate at 340 nm.

8-OHdG assay

The measurement was performed of enzyme-linked immonosorbent assay (ELISA) technique

(ELISA Kit, Elabscience, E-EL-0028). This ELISA kit type is the competitive one. In principle, a fixed amount of a label, the biotinylated detection Ab specifically competes with free 8-OHdG for sample and standard. The bound label 8-OHdG was detected by the addition of the avidin label by horseradish peroxidase, followed by addition of tetra benzin substrate. A blue color formed. Then, 50 microns of stop solution was added. The yellowish color was read at 450 nm.

γ-H2AX assay and observation

The γ-H2AX foci were detected according to Kurnia *et al*. and Chua *et al*. [16,17]. The lymphocytes were fixed in 2 % formaldehyde for 5 minutes. Afterward, the 2 % formaldehyde was removed and replaced with 0.25 % Triton-X, and the lymphocytes were incubated for 5 minutes. Then, the 0.25 % Triton-X was removed and replaced with 1 % BSA and the lymphocytes were incubated again for 15 minutes. Afterward, the 1 % BSA was also removed and replaced, followed by another 15-minute incubation, repeated twice. Finally, the last remaining BSA was removed to as low a level as possible. The first antibody consisted of a mixture of anti-γ-H2AX (mouse anti-phospho-γ-H2AX (Ser139) antibody, ThermoFisher) and 53BP1 antibodies (ThermoFisher; used for control staining) in 1 % BSA in a ratio of 1:500. The first antibody was dropped on the slides and incubated in a dark moist chamber for 45 minutes at 37 °C. The first antibodies were removed, and then the tissue culture was washed with BSA 1 % for 5 minutes; this step was carried out three times. Then, add the second antibody (mixture of Goat Anti-mouse IgG Dylight 488, anti-bityl-Dylight 594 nm, and 4,6-diamidino-2-phenylindole (DAPI)), diluted in 1 % BSA with a ratio of 1:500), and incubated in a dark moist chamber for 30 minutes. Then, the slides were dried using a fan for 10 minutes. To each slide, antifade mounting was applied and a cover glass attached. Then, it was observed with a 100× fluorescence microscope using immersion oil. Fifty foci of γ-H2AX was counted for each sample.

Statistical analysis

The data was analyzed using IBM SPSS Statistics 24.0 software. If the data was normally distributed, the data analysis was continued by ANOVA following by ad hoc test. If, however, data was not normally distributed even after transformation, nonparametric analysis was performed.

RESULTS AND DISCUSSION

Malondialdehyde

As shown in Fig. 2, the gamma irradiation initiated an increase of MDA concentration (lymphocytes and plasma) compared to control. The antioxidants administration reduced MDA formation. Significant decreases were found as the result of the application of dogfruit and tomato extracts and NAC groups. The decline was more significant after the use of tomato extract than after dogfruit extract or NAC use. The identical result is also observed in plasma.
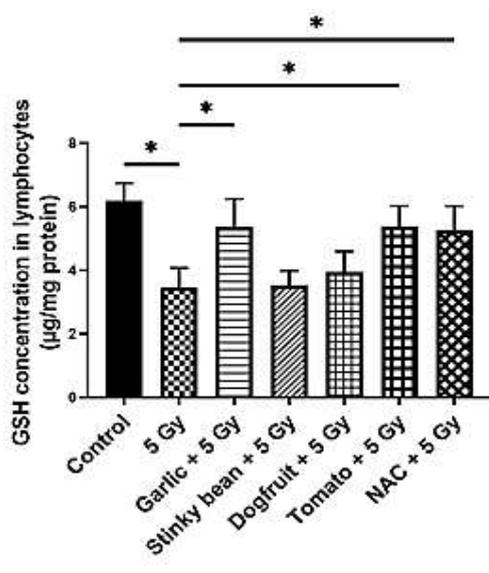
(a)

(b)
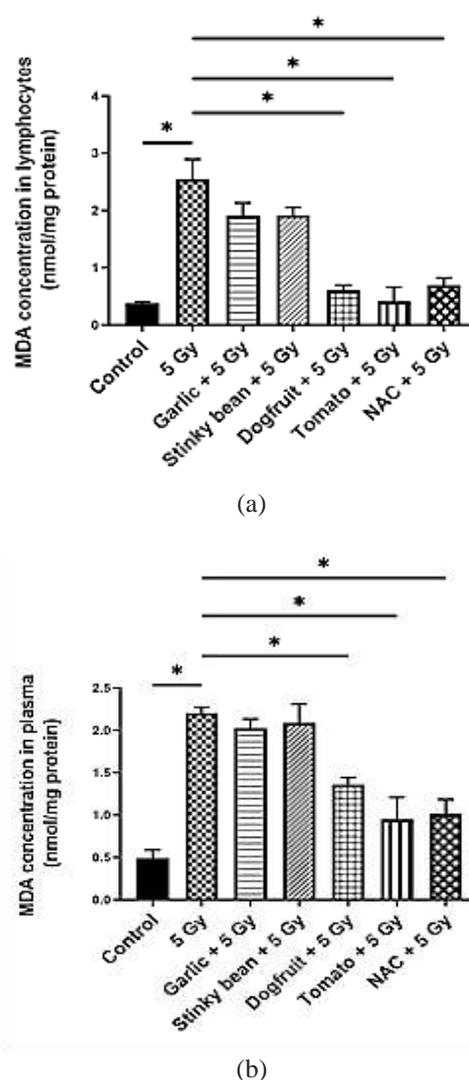
Fig. 2. Effects of gamma irradiation and antioxidant materials on MDA concentration. (a) Lymphocytes; (b) Plasma.
Significant differences are:
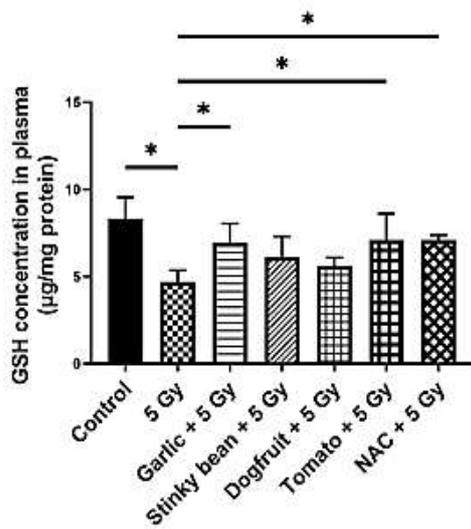∗ P<0.05, compared to the irradiation group (5 Gy).

Glutathione

The effects of gamma irradiation and antioxidant materials on GSH concentration are

shown in Fig. 3. The lowest GSH concentration both in lymphocytes and in plasma were shown by the irradiation group. Antioxidant materials administration led to increases in GSH. A significant increase was observed in the garlic, tomato, and NAC group applications. The highest result was found in tomato extract application.
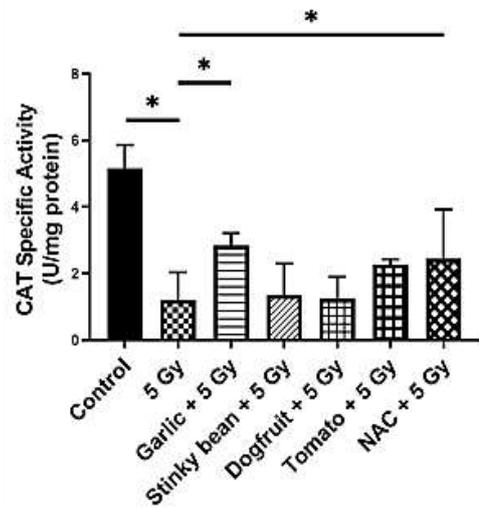

(a)

(b)

Fig. 3. Effects of gamma irradiation and antioxidant materials on GSH concentration. (a) Lymphocytes; (b) Plasma.
Significant differences are:
* $P<0.05$, compared to the irradiation group (5 Gy)

Catalase

The effects of gamma irradiation and antioxidants administration on lymphocytes' CAT specific activity are shown in Fig. 4. The gamma

irradiation significantly reduced CAT's specific activity. The antioxidants administration increased CAT's specific activity, significantly so for the groups treated with garlic extract and NAC.

Fig. 4. Effects of gamma irradiation and antioxidant materials on lymphocytes CAT specific activity.
Significant differences are:
* $P<0.05$, compared to the irradiation group (5 Gy)

Glutathione peroxidase

The gamma irradiation induced a decrease in lymphocyte GPx specific activity compared to control, as shown in Fig. 5. Antioxidant materials administration have led to decrease lymphocyte GPx specific activity compared to irradiation group. A significant increase was seen in the garlic and tomato groups and the more significant effect was found in the garlic group.
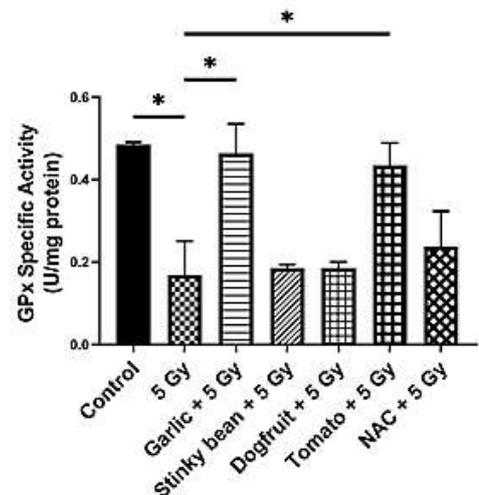
Fig. 5. Effects of gamma irradiation and antioxidant materials on lymphocytes GPx specific activity.
Significant differences are:
* $P<0.05$, compared to the irradiation group (5 Gy)

8-hydroxy-2-deoxyguanosine

The effects of gamma irradiation and antioxidants on 8-OHdG concentration are shown in Fig. 6. The gamma irradiation produced the significant increase of lymphocytes 8-OHdG concentration compared to control. Antioxidants induced a decrease in 8-OHdG concentration compared to the irradiation group. A decrease was shown in the garlic, tomato and NAC groups and highly significant in the garlic group.
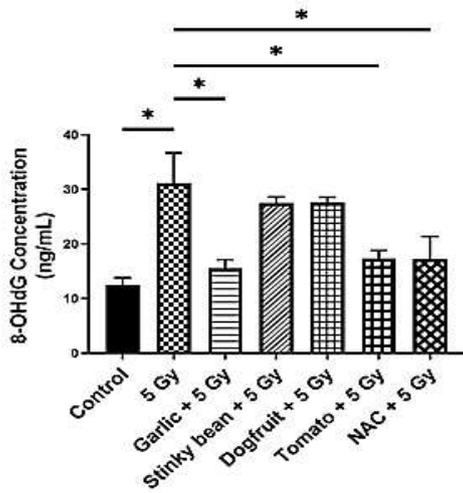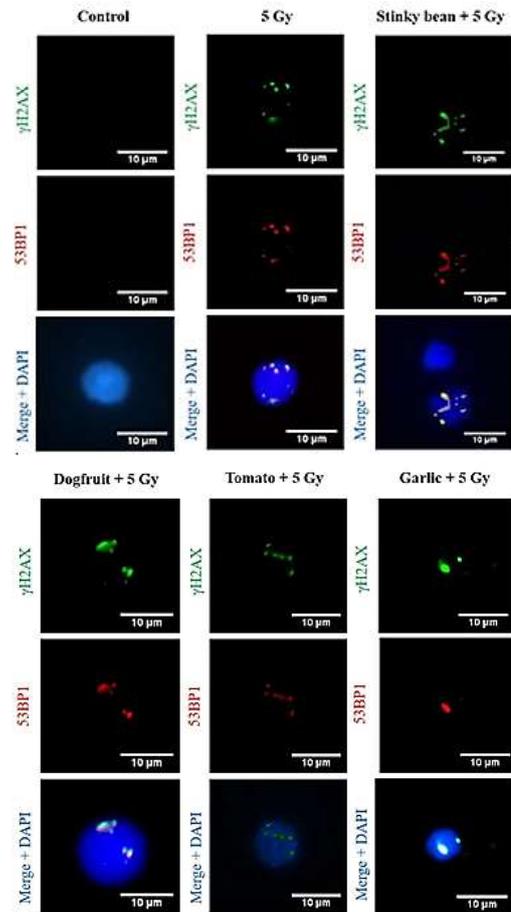
Fig. 6. Effects of gamma irradiation and antioxidant materials on lymphocytes 8-OHdG concentration.
Significant differences are:
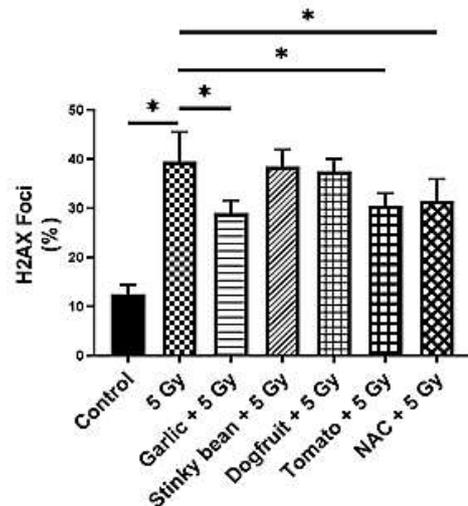* $P < 0.05$, compared to the irradiation group (5 Gy)

γ-H2AX foci

The bright green foci indicate γ-H2AX expression which is the result of bonding between γ-H2AX antibody and secondary antibody (Fig. 7). The effect of gamma irradiation and antioxidant materials on γ-H2AX foci are shown in Fig. 8. The gamma irradiation has led to a significant increase in γ-H2AX foci compared to control. Antioxidants administration were able to decrease γ-H2AX foci compared to irradiation group. A decrease was observed on the garlic, tomato, and NAC groups and was found significant in the garlic group.

Ionizing radiation can produce ROS that cause oxidative stress, which is an imbalance between the formation of free radicals and the availability of antioxidants in cells [23]. The level of cell damage induced by radiation depends on various factors, including radiation exposure doses, antioxidant defense systems and the amount of ROS formed. The radiation generates damages in the membrane and hematopoietic system, increases lipid and

peroxidation, and can affect the antioxidant defense system, resulting in oxidative stress conditions [24].

Fig. 7. Expression of γ-H2AX in lymphocytes.

Fig. 8. Effects of gamma irradiation and antioxidant materials on γ-H2AX foci.
Significant differences are:
* $P < 0.05$, compared to the irradiation group (5 Gy)

The MDA is the end product of lipid peroxidation process [25]. Unsaturated fatty acids

(PUFA) can undergo the process of peroxidation into lipid peroxides which then decompose into MDA. High MDA concentrations indicate an oxidation process in cell membranes [26]. ROS also plays a role in reducing endogenous antioxidants such as GSH, GPx, and CAT. Meanwhile, DNA damage caused by radiation can be detected by increasing the concentration of 8-OHdG and the amount of γ-H2AX foci.

This current study shows that the administration of various antioxidants contained in natural ingredients such as garlic, stinky bean, dog fruits, and tomato reduces the effects of free radicals due to ionizing radiation. Antioxidants administration for eight consecutive days can reduce MDA concentration and increase endogenous antioxidant.

The garlic extract increases GSH levels (lymphocytes and plasma), GPx specific activities, and CAT specific activities; it also reduces 8-OHdG level and formation of γ-H2AX foci. The active ingredients in garlic such as s-allylcysteine, s-allylmercaptocysteine, allicin, and selenium have strong antioxidant potential to counteract free radicals caused by gamma irradiation [12]. The study by Bertrand *et al*., stated that administration of garlic extract in rats was able to significantly increase GSH concentrations and CAT activity in serum induced by radiation [27]. Meanwhile, research conducted by L. Uzun *et al*., showed that garlic extract was able to increase GPx activity in liver tissue [7]. In addition, it is known that garlic prevents the increases of 8-OHdG level in brain tissue and advanced oxidation protein products (AOPP) levels in plasma. S-allyl cysteine has been found in studies to neutralize superoxide ($O_2\bullet$) anion radicals, hydroxyl radicals (OH•), peroxynitrit ($ONOO^-$) anion radicals, and hydrogen peroxide (H_2O_2) [7].

In this current result, stinky bean extract can provide protection against free radicals but not significantly. Meanwhile, the administration of dogfruit extract was able to significantly reduce MDA levels, both in lymphocytes and plasma. Probably, djenkolic acid has strong antioxidant activity. The djenkolic acid molecule will be broken down into a cysteine molecule and a methionine molecule. Cysteine is one of the compounds that can overcome free radicals. Research conducted by Oktrian *et al*. showed that rats given djenkol seed extract had lower MDA concentrations induced CCl_4 [9].

The tomato extract was found to display a significant effect on the overall parameters, except for the increase in CAT activity. Tomato extract can increase endogenous antioxidant capacity such as GSH and GPx, both lymphocytes and plasma. Srinivasan *et al*. stated that administration of lycopene from tomato extract can significantly increase the concentration of GSH and GPx activities induced by radiation. The lycopene antioxidant activity has been demonstrated in both culture cells and animal models [1]. The previous researchers that conducted by Jornet *et al*., found that lycopene given 24 hours before irradiation reduced the structural damage to the salivary glands [28]. Karahan *et al*. stated that lycopene can prevent oxidative DNA damage in diabetic rats. In addition, many studies indicate that lycopene has a positive effect in preventing DNA damage due to radiation, such as the formation of 8-OHdG [29]. Cocate *et al*. suggests that giving carotenoid intake in middle-aged men is positively correlated with decreasing oxidative damage to DNA [30]. The possible mechanisms by which carotenoids quench singlet oxygen ($^1O_2$) and other excited species. $^1O_2$ can bind to the lycopene molecule, converting it to the energy rich triplet state [31].

CONCLUSION

This current study provides evidence that the garlic, dogfruit, and tomato extracts and N-acetylcysteine can significantly protect the body from free radicals caused by gamma irradiation. We recommend further research focused on identifying the active material of those vegetables.

ACKNOWLEDGMENT

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